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Cholinergic Axons in the Rat Ventral Tegmental Area Synapse Preferentially onto Mesoaccumbens Dopamine Neurons

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Abstract

Cholinergic afferents to the ventral tegmental area (VTA) contribute substantially to the regulation of motivated behaviors and the rewarding properties of nicotine. These actions are believed to involve connections with dopamine (DA) neurons projecting to the nucleus accumbens (NAc). However, this direct synaptic link has never been investigated, nor is it known whether cholinergic inputs innervate other populations of DA and GABA neurons, including those projecting to the prefrontal cortex (PFC). We addressed these questions using electron microscopic analysis of retrograde tract-tracing and immunocytochemistry for the vesicular acetylcholine transporter (VAcHT) and for tyrosine hydroxylase (TH) and GABA. In tissue labeled for TH, VAcHT+ terminals frequently synapsed onto DA mesoaccumbens neurons but only seldom contacted DA mesoprefrontal cells. In tissue labeled for GABA, one third of VAcHT+ terminals innervated GABA-labeled dendrites, including both mesoaccumbens and mesoprefrontal populations. VAcHT+ synapses onto DA and mesoaccumbens neurons were more commonly of the asymmetric (presumed excitatory) morphological type, whereas VAcHT+ synapses onto GABA cells were more frequently symmetric (presumed inhibitory or modulatory). These findings suggest that cholinergic inputs to the VTA mediate complex synaptic actions, with a major portion of this effect likely to involve an excitatory influence on DA mesoaccumbens neurons. As such, the results suggest that natural and drug rewards operating through cholinergic afferents to the VTA have a direct synaptic link to the mesoaccumbens DA neurons that modulate approach behaviors.

Keywords

dopamine; GABA; acetylcholine; nucleus accumbens; prefrontal cortex; ultrastructure

Cholinergic projections to the substantia nigra and ventral tegmental area (VTA) derive mainly from the pedunculopontine (PPT) and laterodorsal (LDT) tegmentum (Woolf and Butcher, 1986; Hallanger and Wainer, 1988; Lavoie and Parent, 1994; Oakman et al., 1995; Forster and Blaha, 2000) and provide an important substrate for the reinforcing properties of nicotine and the pathophysiology of addiction as well as mental disorders such as schizophrenia (Nisell et al., 1994; Yeomans, 1995; Nestler, 2001; Corrigall et al., 2002; Mansvelder and McGehee, 2002; Dani, 2003). Within the VTA, acetylcholine and agonists of nicotinic and muscarinic receptors typically increase the activity of dopamine (DA) and GABA cells, promote bursting, and phasically increase efflux in terminal regions (Corrigall et al., 1994; Nisell et al., 1994; Westerink et al., 1996; Westerink et al., 1998; Forster and Blaha, 2000; Grillner and Svensson, 2000; Gronier et al., 2000; Yin and French, 2000).

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Burst firing in DA neurons provides an important physiological mechanism for signaling behaviorally relevant events and enhancing DA release in target areas (Suaud-Chagny et al., 1992; Schultz et al., 1997; Paladini and Tepper, 1999; Redgrave et al., 1999). Hence, cholinergic inputs from the mesopontine tegmentum may provide an important source of salience information for DA neurons (Inglis and Winn, 1995; Miller et al., 2002). Nicotine has particularly been shown to influence the release of DA in the nucleus accumbens (NAc) (Nisell et al., 1994), consistent with the role of the mesoaccumbens pathway in reward behaviors (Koob, 1996; Redgrave et al., 1999; Nestler, 2001). VTA DA neurons also contribute to cognitive functions via projections to the prefrontal cortex (PFC) (Goldman-Rakic, 1995), and nicotine also regulates mesoprefrontal DA release (George et al., 2000).

The forebrain projections of the VTA (Swanson, 1982) (Loughlin and Fallon, 1984) arise from both DA and GABA cells (Van Bockstaele and Pickel, 1995; Carr and Sesack, 2000) with related but discrete functions (Laviolette and van der Kooy, 2001; Lee et al., 2001; Steffensen et al., 2001). The VTA projections to the PFC and NAc arise from separate, essentially non-collateralized cell populations, as established by multiple retrograde tract-tracing studies (Fallon, 1981; Swanson, 1982; Loughlin and Fallon, 1984). Within these populations, there is additional functional evidence for the segregation of DA mesoaccumbens and mesoprefrontal neurons, based on their distinct neurochemical features and response to environmental stimuli (Thierry, 1976; Deutch et al., 1991; White, 1996; Tzschentke, 2001). These observations suggest that different populations of VTA cells might receive distinct patterns of innervation from afferent sources, a hypothesis supported by our studies of inputs from the PFC and LDT (Carr and Sesack, 2000; Omelchenko and Sesack, 2005). A previous investigation of cholinergic terminals in the VTA (Garzon et al., 1999) showed that the synaptic targets included both cells labeled for the DA transporter and unlabeled neurons. In that study, it was further suggested that cholinergic inputs preferentially innervate mesoprefrontal DA neurons as defined by their low content of DA transporter. However, this hypothesis is challenged by neurochemical experiments suggesting that activation of cholinergic receptors influences both mesoaccumbens and mesoprefrontal DA cells (Corrigall et al., 1994; Westerink et al., 1996; Westerink et al., 1998; Forster and Blaha, 2000; George et al., 2000; Gronier et al., 2000).

Hence, it is essential to examine the precise cellular targets of cholinergic inputs to the VTA to better define the anatomical substrates of behavioral control mediated by this important regulatory pathway, including its contribution to drug seeking. Although acetylcholine can influence cell activity via presynaptic receptors (Grillner and Svensson, 2000; Jones and Wonnacott, 2004), the present study was designed to address the direct synaptic connections of cholinergic afferents to identified cell populations in the VTA. Based on available physiological and pharmacological data, we hypothesized that both mesoaccumbens and mesoprefrontal DA neurons would receive cholinergic input. Although comparable physiological and neurochemical data for the GABA component of these populations are not available, prior findings (Garzon et al., 1999) are consistent with direct cholinergic inputs to GABA cells as well. Hence, we also hypothesized that both major populations of VTA GABA neurons would receive cholinergic innervation.

These issues were addressed by electron microscopic examination of the VTA in animals receiving injections of the retrograde tracer FluoroGold (FG) into either the PFC or NAc. Immunoperoxidase was used to visualize both the vesicular acetylcholine transporter (VACHT) as a marker for cholinergic terminals (Garzon et al., 1999) and FG in the soma and dendrites of mesoaccumbens and mesoprefrontal neurons (Carr and Sesack, 2000; Omelchenko and Sesack, 2005). Immunogold-silver labeling was then used to identify tyrosine hydroxylase (TH) or GABA phenotypes within the VTA.

MATERIALS AND METHODS

Subjects and surgeries

The experiments were performed on eight adult male Sprague-Dawley rats (Hilltop Lab Animals Inc, 330-360 g) in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and with the approval of the Institutional Animal Care and Use Committee at the University of Pittsburgh. One naive rat was used to determine the optimal dilution of the antibody directed against VAcHT. The remaining seven animals were used for tract-tracing. Rats were deeply anesthetized with an i.m. injection of 34 mg/kg ketamine, 1 mg/kg acepromazine, and 7 mg/kg xylazine and placed in a stereotaxic apparatus. Animals then received bilateral injections of FG (Fluorochrome, Denver, CO) into the NAc (4 animals) or into the PFC (3 animals). FG was dissolved as a 1% solution in 100 mM cacodylate buffer (pH 7.5) and was iontophoretically delivered for 20 min (+5 μ A pulsed 10 sec on/off) through glass micropipettes with 75 μ m tip diameters. The coordinates for the injections were chosen according to the atlas of Paxinos and Watson (Paxinos and Watson, 1998). We used two injections per hemisphere to improve the filling of each area. The coordinates for NAc injections were 1.7 mm anterior to Bregma, 2.5 mm lateral to midline and 6.8 and 7.7 mm ventral to the skull surface, with pipettes lowered at a 10° angle in the coronal plane. The coordinates for PFC injections were 3.0 mm anterior to Bregma, 0.9 mm lateral to midline, and 4 and 5 mm ventral to the skull surface, with pipettes lowered straight down. After the injections, the pipettes were left in place for 5 minutes before extracting them.

Following 5-14 days survival, the animals were anesthetized with pentobarbital (60 mg/kg, i.p., with supplemental doses if necessary) and treated for 15 min with 1 g/kg i.p. of the zinc chelator diethyldithiocarbamic acid (Sigma, St. Louis, MO) to prevent silver intensification of endogenous zinc (Veznedaroglu and Milner, 1992). The animals were killed by a transcardial perfusion of heparin saline (Elkins-Sinn, NJ; 1000 U/ml), followed by 50 ml of 3.75% acrolein and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4) and 200-400 ml of 2% paraformaldehyde in 0.1 M PB. The brains were removed and cut into blocks containing the PFC and NAc or the VTA. The blocks were post-fixed in 2% paraformaldehyde for 0.5-1 hr and sectioned in the coronal plane on a vibratome. The serial sections (50 μ m) were collected in PB and incubated in 1% sodium borohydride in PB for 30 min in order to terminate fixation. Sections were subsequently rinsed in PB.

Immunocytochemistry

The present investigation employed a triple-immunolabeling procedure that is similar to the one used in our prior publications (Carr and Sesack, 2000; Omelchenko and Sesack, 2005). In this case, immunoperoxidase was used to detect both the presence of the retrograde tracer FG in soma and dendrites (Carr and Sesack, 2000; Omelchenko and Sesack, 2005) and the specific and sensitive cholinergic marker VAcHT in axons (Garzon et al., 1999). Immunogold-silver labeling for the catecholamine synthetic enzyme TH or for GABA itself was then used to identify dendrites as belonging to DA or GABA neurons.

The primary antibodies directed against FG, TH and GABA have been used in our prior publications (Sesack et al., 1995; Carr and Sesack, 2000; Carr and Sesack, 2000; Omelchenko and Sesack, 2005) and those of other laboratories (Van Bockstaele and Pickel, 1995; Aston-Jones et al., 2004; Waselus et al., 2005). The specificity of the rabbit polyclonal FG antibody (Chemicon #AB153) is demonstrated by the complete absence of staining in brain regions that are known not to project to the area of the injection site. The mouse monoclonal antibody directed against TH (Chemicon #MAB318) recognizes a 59-61 kDa protein from the outside of the N-terminus of TH purified from PC12 cells and has been

shown by western blot analysis to not recognize other monoamine synthetic enzymes (manufacturer's information). The mouse monoclonal antibody against GABA (Sigma #A-0310) was raised against purified GABA conjugated to bovine serum albumin (BSA) and has been shown by dot blot immunoassay not to recognize several closely related amino acids (manufacturer's information). The antibody directed against VACHT (Immunostar #24286) was raised in goat and generated against a synthetic peptide that corresponds to amino acids 511-530 from the C-terminus of the cloned rat VACHT. In transfected cell lines, this antibody has been shown not to cross react with vesicular monoamine transporters (Arvidsson et al., 1997), a result which is consistent with the absence of immunoreactivity for VACHT in soma or dendrites within the VTA (Garzon et al., 1999) (present study). Additional evidence for the specificity of the VACHT antibody includes labeling of cells transfected with the VACHT cDNA but not untransfected cells, selective labeling of cholinergic PC12 cells and spinal motor neurons in primary cultures, colocalization with the cholinergic synthetic enzyme choline acetyltransferase, and preadsorption with the immunizing peptide (Arvidsson et al., 1997). The same antibody has been used in a previous study of the cholinergic innervation of the VTA (Garzon et al., 1999).

The distinct neuronal compartmentalization of FG and VACHT was established by analysis of control tissue sections singly labeled for each protein and is in full agreement with the available literature (Schmued and Fallon, 1986; Novikova et al., 1997; Garzon, 1999 #2695). In our prior study of the LDT projection (Omelchenko and Sesack, 2005), we demonstrated that VTA sections labeled only for FG retrogradely transported from the NAc or the PFC contained immunoperoxidase for FG only within cell bodies and dendrites and not axons. Similarly, in the current study, examination of anterior/middle VTA sections labeled only for VACHT revealed peroxidase product for this marker only within axons and axon terminals.

Immunolabeling procedures were performed on free-floating sections at room temperature with constant shaking, and all immunoreagent incubations were followed by extensive rinses in buffer. After being rinsed in 0.1 M tris-buffered saline (TBS; pH 7.6), sections were placed for 30 min in a blocking solution containing 1% BSA and 3% normal goat serum in 0.1 M TBS. To improve penetration for immunoreagents, Triton X-100 (Sigma) was added to the blocking solution at 0.04% for electron microscopy or at 0.2% for light microscopy. Sections were then incubated for 12-15 hrs in a mixture of three primary antibodies diluted in the blocking solution: rabbit anti-FG (1:2000-4000; Chemicon, Temecula, CA), goat anti-VACHT (1:16,000-40,000; ImmunoStar, Hudson, WI), and either mouse anti-TH (1:5000; Chemicon) or mouse anti-GABA (1:2000; Sigma). Sections were then incubated for 30 min in the blocking solution containing a mixture of biotinylated secondary antibodies: donkey anti-rabbit and donkey anti-goat IgGs (both 1:400; Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were then incubated for 30 min in 1:100 avidin-biotin peroxidase complex (Vectastain Elite kit; Vector Laboratories). The peroxidase reaction was visualized by incubating the sections in 0.022% diaminobenzidine (Sigma) and 0.003% hydrogen peroxide in TBS for 3-3.5 min, and the reaction was stopped by several rinses in TBS. Sections for light microscopic examination of VACHT or FG injection and transport were processed using single labeling with one primary and one secondary antibody and the avidin-biotin peroxidase method.

After immunoperoxidase labeling, tissue was processed for immunogold-silver labeling of TH or GABA. Sections were placed for 30 min in a second blocking solution containing 0.8% BSA, 0.1% fish gelatin, and 3% normal donkey serum in 10 mM phosphate buffered saline (PBS; pH 7.4). The sections were then incubated for 12-15 hr in this solution to which was added 1:50 donkey anti-mouse IgG conjugated to 1 nm gold particles (Electron Microscopy Sciences, Washington, PA). After several rinses in the blocking solution and in

PBS, the sections were postfixated by 2% glutaraldehyde in PBS for 10 min and rinsed again in PBS and then in 0.2 M sodium citrate buffer, pH 7.4. To enhance the size of the gold particles, the sections were placed in silver solution (Amersham) for 4-6 min and then rinsed in citrate buffer.

Tissue preparation for light and electron microscopy

Sections for light microscopy were mounted onto glass slides. After drying, they were dehydrated in graded alcohol solutions, defatted in xylene, and coverslipped with DPX. Sections were examined by bright field microscopy to verify successful injection sites and transport of FG and to illustrate the density of VAcHT in the ventral midbrain. Digital photomicrographs obtained from these sections were processed in Adobe Photoshop to match brightness and contrast.

Sections for electron microscopy were postfixated in 2% osmium tetroxide in PB for 1 hr. Sections were then dehydrated through a series of alcohol solutions with increasing concentrations, immersed in propylene oxide, and then left overnight in equal volumes of propylene oxide and epoxy resin (EM bed 812; Electron Microscopy Sciences). Afterwards, sections were placed in straight epoxy resin for 2 hr and then embedded between sheets of commercial polymer film that were placed in an oven for 48 hr at 60°C to harden the epoxy resin.

Plasticized sections were photographed to document the exact position within the parabrachial and paranigral subdivisions of the anterior/middle VTA where ultrathin sections were taken. These sections were cut from the outermost surface of the vibratome sections that contained the interface zone between tissue and plastic resin. Ribbons of 3-7 consecutive sections were collected onto mesh grids and counterstained with uranyl acetate and lead citrate before examination on a transmission electron microscope: Zeiss 902 (Oberkochen, Germany) or FEI Morgagni (Hillsboro, Oregon). Analog photomicrographs from the Zeiss microscope were printed to comparable contrast and illumination. Digital micrographs captured from the FEI microscope using an AMT XP-60 digital camera (Danvers, MA) were similarly adjusted using Adobe Photoshop.

Ultrastructural analysis

Systematic examination of the tissue surface was performed at 12,000-20,000X magnification. Neuronal elements were identified according to Peters (Peters et al., 1991). In animals receiving FG injections into the NAc, 189,819 μm^2 of tissue was examined from TH-labeled sections and 641,300 μm^2 from GABA-labeled sections. In animals receiving FG injections into the PFC, 534,669 μm^2 of tissue was examined from TH-labeled sections and 316,869 μm^2 from GABA-labeled sections. The area examined was calculated by multiplying the number of grid squares analyzed, the known boundaries of the grid mesh (3,025 μm^2), and an estimate of the percentage of each square that contained tissue as opposed to resin. All axon terminals labeled by immunoperoxidase for VAcHT were photographed at 18,000-20,000X, and the synaptic contacts of these terminals were evaluated.

In order to minimize false negative findings due to technical limitations, we confined the analysis to the anterior-middle VTA regions that have the most extensive retrograde transport of FG. To further limit the incidence of false negative results, we also performed the analysis close to the outermost surface of the tissue where it interfaced with epoxy resin. Moreover, images of immunoperoxidase were analyzed only if profiles labeled by immunogold-silver were also evident within the same photographic field (approximately 13.8 μm^2). This ensured that the less sensitive immunogold reagents had penetrated at least

to this depth. Serial sections were analyzed in cases where immunoperoxidase labeling for FG required verification or immunogold-silver labeling was sparse. Profiles were defined as gold-silver labeled if they contained at least 3 particles in one section; in most cases, labeled dendrites contained many more than 3 particles.

Statistical analysis

Categorical differences in the extent to which VACHT+ profiles formed symmetric versus asymmetric synapses or contacted different VTA cell populations were compared statistically using Fisher's exact test (Matthews and Farewell, 1996) unless data sets contained zero values. Significance was established when P values were less than 0.05, two-tailed.

RESULTS

Light microscopy

FG injections into the NAc or PFC were performed in a manner similar to that used in previous studies and produced similar results (Carr and Sesack, 2000; Omelchenko and Sesack, 2005). Within the NAc, FG injection sites included both the core and shell subdivisions at mid rostro-caudal levels (Fig. 1A), whereas FG injections in the PFC were placed within prelimbic and infralimbic areas in rostral and middle parts of this structure (Fig. 1B). Retrograde transport of FG was observed within the soma and dendrites of VTA neurons (Fig. 1C,D) and notably involved fewer neurons following FG injections into the PFC.

In the light microscope, VACHT+ profiles labeled by immunoperoxidase product were identified as axons throughout all regions of the VTA (Fig. 1E-G), including those that contained mesoprefrontal and mesoaccumbens neurons. Within the ventral midbrain, the density of VACHT+ profiles was most profound within the interpeduncular nucleus (Fig. 1E) and more moderate within the VTA (Fig. 1E-G). Labeling was weak or absent in the substantia nigra zona reticulata (not shown) and in prominent white matter tracts surrounding the VTA. The majority of VACHT+ profiles appeared as separate puncta, most likely representing axon boutons; in some cases, multiple labeled boutons appeared to lie along individual axons (Fig. 1G).

Electron microscopy

Cholinergic input onto VTA neurons—Immunoperoxidase for VACHT was observed as a dark flocculent product within axons and axon terminals (Fig. 2) whose morphological features resembled those previously described for cholinergic fibers in this region (Bolam et al., 1991; Garzon et al., 1999). Labeled terminals contained tightly packed round and oval vesicles, occasional mitochondria, and infrequent dense cored vesicles. In tissue sections labeled for GABA, some VACHT-immunoreactive axons were dually labeled by immunogold-silver for GABA (Fig 2C).

A substantial number of VACHT-labeled terminals contacted dendrites or soma (770/1021, 75%), and synapses were formed at 44% of these contacts observed in single sections (335/770; Table 1). The synaptic targets were predominantly dendrites of variable caliber (Figs. 2-5), although some examples of axo-somatic synapses were also observed (Fig. 4C). Most VACHT+ terminals (242/335, 72%) formed Gray's Type I synapses (Gray, 1959) with thickened postsynaptic densities and widened synaptic clefts (i.e. asymmetric; Fig. 2A). The thin postsynaptic densities and narrower synaptic clefts exhibited by the remaining synapses were consistent with Gray's Type II morphology (i.e. symmetric; Fig. 2B,C).

VACHT+ synapses onto TH- and GABA-labeled neurons—Within tissue sections immunostained for TH, 43% (88/203) of synapses formed by VACHT+ terminals were onto TH-labeled dendrites or soma (Fig. 3A,C,D); the remaining synapses were onto targets that did not contain TH (Table 1). VACHT+ synapses that innervated TH dendrites were mostly of the asymmetric type (Fig. 3A; 73/88, 83%), although some synapses with symmetric morphology were also observed (Fig. 3C,D). Within the GABA immunostained tissue sections from the same animals, 33% (44/132) of VACHT+ synapses innervated GABA-labeled neurons (Fig. 3B,E); the rest contacted dendrites that were unlabeled by immunogold-silver. The majority of VACHT labeled terminals synapsing onto GABA-immunoreactive profiles were also of the asymmetric type (Fig. 3B; 24/44, 55%). However, in this case, the proportion of symmetric synapses (Fig. 3E) was notably higher than observed onto TH-labeled structures. The extent to which VACHT+ axons were seen to contact TH or GABA-labeled structures at asymmetric versus symmetric synapses was significantly different ($p = 0.006$).

VACHT+ synapses onto mesoaccumbens and mesoprefrontal neurons—

Analysis of VTA dendrites containing immunoreactivity for FG (Table 1) revealed that VACHT-labeled terminals synapsed onto both mesoaccumbens (Fig. 4; 35/193, 18%) and mesoprefrontal (Fig. 5; 12/142, 8%) populations, with a significantly greater number of the former contacts ($p = 0.016$). The majority of VACHT+ synapses onto the dendrites of mesoaccumbens neurons were of the asymmetric type (Fig. 4A,C-E; 32/35, 91%); the rest were defined as symmetric (Fig. 4B,F,G). This observation contrasted markedly with the mesoprefrontal population, for which only a minority of synapses formed by VACHT-labeled terminals were asymmetric (Fig. 5A,C; 4/12, 25%), and the majority were of the symmetric type (Fig. 5B,D). This difference in the extent to which VACHT+ axons were observed to contact mesoaccumbens and mesoprefrontal neurons via asymmetric versus symmetric synapses was significant ($p = 0.02$).

In TH-labeled tissue sections, TH immunoreactivity was observed within a considerable number of the mesoaccumbens dendrites (21/25, 84%) and a lower proportion of the mesoprefrontal neurons (3/6, 50%) that were synaptically innervated by VACHT-labeled terminals (Table 1). The majority (20/21, 95%) of synapses onto TH-immunoreactive mesoaccumbens dendrites were of the asymmetric (Fig. 4A,C) versus the symmetric type (Fig. 4B), whereas the less frequent contacts onto TH-labeled mesoprefrontal dendrites included one asymmetric (Fig. 5A) and two symmetric (Fig. 5B) synapses. Analysis of GABA-immunostained tissue sections showed that GABA immunolabeling occurred within both the mesoaccumbens (7/10, 70%) and the mesoprefrontal populations (2/6, 33%) that were synaptically contacted by VACHT+ axons. The majority of VACHT-labeled synapses onto mesoaccumbens GABA cells were of the asymmetric type (Fig. 4D,E; 6/7, 86%), and only one symmetric synapse was detected (Fig. 4F,G). Conversely, we observed only symmetric VACHT+ synapses onto mesoprefrontal GABA cells (Fig. 5D).

DISCUSSION

The present data provide the first morphological evidence for a substantial direct input from cholinergic afferents to DA cells of the mesoaccumbens pathway, consistent with hypotheses that such connectivity underlies the rewarding and addictive properties of nicotine (Corrigall et al., 1994; Mansvelder and McGehee, 2002). The findings of a substantial cholinergic innervation of mesoaccumbens DA neurons agree with physiological and neurochemical measurements and suggest that cholinergic axons contribute a considerable proportion of the synaptic input to this cell population (Omelchenko and Sesack, 2005). The present results further demonstrate that cholinergic afferents to the VTA provide lower frequency synaptic input to mesoprefrontal DA neurons and to GABA cells of

both populations. The observation that cholinergic terminals form both symmetric and asymmetric synapses is consistent with recent reports that acetylcholine is colocalized with glutamate or GABA and suggests that this transmitter system is likely to evoke multiple, complex effects in VTA neurons (Fig. 6).

Methodological Considerations

False negative results in synapse counting comprise the major technical limitation for studies of this type that rely on tracer injections and electron microscopic immunocytochemistry, as we have discussed in our prior publications (Carr and Sesack, 2000; Omelchenko and Sesack, 2005). Briefly, most injected tracers do not label the entirety of a population of interest. Moreover, limited penetration of antibodies in tissue processed with low detergent levels can lead to sparse or absent distribution of immunoproducts within profiles that actually do contain the antigen of interest.

For the present study, the use of immunolabeling for VACHT to identify cholinergic inputs to the VTA provided an advantage over prior investigations using anterograde tract-tracing (Omelchenko and Sesack, 2005), in that a greater proportion of the cholinergic afferents was likely to be represented, including possibly minor projections from outside the brainstem tegmentum. Furthermore, the considerable number of profiles shown to be synaptically innervated by cholinergic terminals in the current study suggests that all the VTA populations examined could be shown to receive cholinergic innervation if such inputs existed. We acknowledge that some false negative results may have lead to an underestimation of the cholinergic inputs generally. However, the data are likely to reflect the approximate organization of cholinergic synaptic inputs onto defined VTA cell populations

Cholinergic terminals within the VTA

Our finding of numerous VACHT-labeled unmyelinated axons and axon terminals forming predominantly asymmetric axo-dendritic synapses within the VTA supports previous reports of cholinergic innervation to this and adjacent brain regions (Hallanger et al., 1990; Bolam et al., 1991; Garzon et al., 1999; Garzon and Pickel, 2000). However, the present observation of 25% symmetric synapses is inconsistent with prior reports within the ventral midbrain (Bolam et al., 1991; Garzon et al., 1999). Although some part of this discrepancy may reflect variation in the criteria used to identify synapse type, it is important to note that cholinergic axons forming symmetric synapses have been described in other targets of the brainstem tegmentum (Hallanger et al., 1990; Garzon and Pickel, 2000).

The morphology and synaptology of cholinergic axons within the VTA are similar to those observed for the innervation arising from the LTD (Omelchenko and Sesack, 2005), suggesting that terminals containing acetylcholine comprise a substantial portion of the LTD innervation to the VTA. However, additional cholinergic axons are likely to arise from the caudal PPT (Oakman et al., 1995) and may derive from minor projections outside the brainstem tegmentum, most notably the medial habenula and diagonal band (Phillipson, 1979; Ichikawa et al., 1997; Geisler and Zahm, 2005). Moreover, a profound influence of cholinergic inputs to the ventral midbrain is suggested by observations that individual axons form multiple synapses onto the same dendrites (Bolam et al., 1991).

It is an assumption that the predominant class of cholinergic terminals forming asymmetric synapses is likely to mediate an excitatory action (Carlin, 1980). Although this association between morphology and physiology is only correlative, it is supported by electrophysiological reports that stimulation of the mesopontine tegmentum induces burst firing in midbrain DA neurons (Lokwan et al., 1999) and by neurochemical studies showing

that stimulation of the LDT evokes forebrain DA release via a mechanism involving cholinergic receptors in the midbrain (Forster and Blaha, 2000). Conversely, the suggestion of likely inhibitory and/or modulatory actions induced by cholinergic terminals forming symmetric synapses (Carlin, 1980) is supported by reports of inhibitory and mixed physiological effects produced by acetylcholine in the VTA (Fiorillo and Williams, 2000; Erhardt et al., 2002).

The morphology of synaptic contacts formed by cholinergic VTA axons may also correlate with the receptors expressed postsynaptically. Cholinergic receptors, including those within the midbrain, are highly diverse both in structure and function and include several classes of nicotinic and muscarinic types (Rathouz et al., 1995; Charpantier et al., 1998; Fiorillo and Williams, 2000; Klink et al., 2001; Azam et al., 2002). Moreover, these receptors may also be expressed presynaptically and alter the release of glutamate and GABA, consistent with extrasynaptic actions of acetylcholine (Grillner et al., 1999; Grillner et al., 2000; Grillner and Svensson, 2000; Erhardt et al., 2002; Jones and Wonnacott, 2004). Hence, a full understanding of the functions of this important transmitter will ultimately require studies localizing cholinergic receptors within the VTA.

The synapse types formed by cholinergic terminals in the VTA are also likely to reflect the colocalization of other transmitters in these axons, in particular glutamate and GABA (Bevan and Bolam, 1995; Clarke et al., 1997; Jia et al., 2003). Our observation of GABA immunoreactivity in some cholinergic terminals in the VTA is consistent with these findings. Moreover, a prior study of the monkey VTA reported that nearly all inputs from the PPT contained either glutamate or GABA (Charara et al., 1996), suggesting that all cholinergic afferents to the midbrain may colocalize one of these two transmitters.

VTA neuronal populations innervated by cholinergic terminals

The observation of at least some synaptic inputs of cholinergic axons onto each of the neuronal populations investigated (Fig. 6) suggests that cholinergic afferents play an important and complex role in VTA cell regulation. The relative abundance of inputs to DA as compared to GABA neurons agrees with previous studies of the VTA (Garzon et al., 1999) and is consistent with cell counts indicating that DA neurons account for 64% of the overall population in this region (Swanson, 1982).

The finding of cholinergic inputs to both mesoaccumbens and mesoprefrontal DA populations agrees with our initial hypothesis. The heavier cholinergic innervation of mesoaccumbens cells may, in part, reflect both the larger size and the relative abundance of the DA phenotype in the mesoaccumbens versus the mesoprefrontal cell populations (Swanson, 1982). However, the striking observation of a four times greater percentage of cholinergic inputs to mesoaccumbens versus mesoprefrontal DA cells indicates a clear preference for the accumbens-projecting DA neurons. This finding contradicts a previous hypothesis of a greater cholinergic innervation to mesoprefrontal DA cells (Garzon et al., 1999) based on a low intensity of labeling for the DA transporter in the target neurons. However, the reported absence of the DA transporter from most cortical DA axons (Sesack et al., 1998) suggests that the transporter is probably not an appropriate marker for the mesoprefrontal DA population. Instead, the greater cholinergic input to cells with low DA transporter levels may reflect a differential innervation of neurons projecting to the NAc shell versus the core (Nirenberg et al., 1997; Jones et al., 1998), which were not distinguished in the present study.

The observation of direct cholinergic inputs to GABA VTA neurons also matches our initial hypothesis and previous reports of cholinergic synapses onto non-DA cells (Garzon et al., 1999). The similarity in cholinergic inputs to DA and GABA neurons with parallel forebrain

projections suggests that these populations may have correspondingly similar cholinergic regulation despite their distinct transmitter phenotypes and differential innervation by the PFC (Carr and Sesack, 2000). In addition, the possibility that VTA GABA neurons also regulate neighboring DA cells through local collaterals (Johnson and North, 1992) suggests that the comparable cholinergic input to these cell populations may function to produce both direct and indirect actions on DA neurons.

The finding of statistical differences in the extent to which cholinergic axons form asymmetric (presumably excitatory) versus symmetric (presumably inhibitory or modulatory) synapses onto different VTA cell populations suggests a rather complex organization of this circuitry. The preferential innervation of DA neurons and mesoaccumbens populations by presumed excitatory synapses and the greater targeting of GABA cells and mesoprefrontal neurons by presumed inhibitory or modulatory synapses suggest that acetylcholine mediates disparate physiological responses in different neuronal circuits. Additional studies addressing these issues are required to more precisely characterize the physiological actions of cholinergic inputs on specific VTA neuronal groups.

Functional Considerations

The cholinergic projections of the mesopontine tegmentum, the main source of cholinergic input to the VTA, function in the control of locomotion, arousal, and sleep-wake cycles (Steriade and McCarley, 1990; Winn et al., 1997), although the exact function of the projection to the VTA is not fully understood. The brainstem tegmentum may participate in a larger integration system for regulating behavioral state (Oakman et al., 1995) and for communicating the salience properties of environmental stimuli (Lokwan et al., 1999; Miller et al., 2002). In this regard, the current data showing preferential cholinergic innervation of mesoaccumbens DA neurons provide credible anatomical substrates for the facilitation of motoric behaviors related to nicotine self-administration and for some of the plasticity changes associated with addiction to nicotine and other drugs of abuse (Nisell et al., 1994; Lanca et al., 2000; Nestler, 2001; Corrigall et al., 2002; Mansvelder and McGehee, 2002; Miller et al., 2002).

The present demonstration of cholinergic inputs to mesoprefrontal DA cells also suggest a previously unappreciated mechanism whereby acetylcholine may influence cognitive function by altering cortical DA levels (Goldman-Rakic, 1995; Winn, 1998; Sesack et al., 2003). The cognitive enhancing effects of nicotine are well established and presumed to be mediated via actions within the cerebral cortex (Rezvani and Levin, 2001; Newhouse et al., 2004). However, it would be interesting in future studies to test whether nicotine's effects on cognition, and its use in the treatment of disorders characterized by cognitive impairment (Levin and Rezvani, 2000; Newhouse et al., 2004), might also involve its actions on VTA DA neurons with projections to the PFC.

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Figure 1.

Light microscopic images of rat coronal brain sections. Panels A and B show injections of FG into the NAc or PFC. The corresponding retrograde transport of FG to the VTA is shown in panels C and D. Inserts illustrate the transport of FG into soma and dendrites (arrows indicate the same cells). Panels E-G illustrate peroxidase labeling for VAcHT in the VTA and adjacent interpeduncular nucleus (IP). Although VAcHT+ fibers are notably denser in the IP than in the VTA, VAcHT axons in the latter structure viewed at higher magnification are markedly beaded and appear to form chains of varicosities (small arrows in G). Abbreviations: ac, anterior commissure; cc, corpus callosum; fm, forceps minor; fr, fasciculus retroflexus; LV, lateral ventricle; ml, medial lemniscus; mp, mammillary peduncle; rs, rhinal sulcus. Scale bar represents 1 mm in A-B, 500 μ m in C-D, 62.5 in inserts, 125 μ m in E-F, and 31.25 μ m in G.

Figure 2.

Electron micrographs of the rat VTA demonstrating immunoperoxidase labeled VAcHT-positive axon terminals (VT) forming synapses with asymmetric (white arrow) or symmetric (black arrows) morphology onto unlabeled dendrites (ud). One VT terminal also shows immunogold-silver labeling for GABA (GABA/VT in C), and a GABA-labeled dendrite appears in the adjacent neuropil (GABA-d in B). Scale bar, 0.33 μ m.

Figure 3. Electron micrographs showing asymmetric (white arrows) or symmetric (black arrows) synapses formed by VChT-positive terminals (VT) onto dendrites singly labeled by immunogold-silver for TH (TH-d) or GABA (GABA-d). The GABA-d in B receives additional synaptic input from a GABA-labeled terminal (GABA-t). The TH-d in C is shown in a serial section in D to verify the TH labeling. Scale bar, 0.33 μ m.

Figure 4.

Electron micrographs of the VTA from rats receiving FG injections into the NAc. VChT-immunoreactive terminals (VT) form asymmetric (white arrows) or symmetric (black arrows) synapses onto soma (s) or dendrites (d) dually labeled by immunoperoxidase for FG and immunogold-silver for TH (FG+TH) or GABA (FG+GABA). In C, the VT synapses onto a FG+TH soma, as evidenced by the presence of a nucleus (n). The FG+GABA-d in F contains a low level of GABA labeling and FG concentrated in a lysosome (arrowhead). The serial section in G shows the synapse formed by the VT. Scale bar, 0.33 μm for A-C and E-G; 0.5 μm for D.

Figure 5.

Electron micrographs of the VTA from rats receiving FG injections into the PFC. VACHT-labeled axon terminals (VT) form asymmetric (white arrows) or symmetric synapses (black arrows) onto dendrites dually-labeled for FG and TH (FG+TH-d) or FG and GABA (FG+GABA-d) or singly-labeled for FG (FG-d). In B, sparse immunogold-silver labeling for TH is indicated by small arrows. Arrowheads indicate diffuse FG in the cytoplasm (B) or concentrated in a lysosome (D). Scale bar, 0.33 μm for A, B, D; 0.40 μm for C.

Figure 6.

Schematic drawing comparing the asymmetric (presumed excitatory) synapses in white and symmetric (presumed modulatory/inhibitory) synapses in black formed by cholinergic axons presumably coming from the mesopontine tegmentum onto identified cell populations of DA (D) and GABA (G) VTA neurons. The thickness of cholinergic axons is weighted to reflect the approximate frequency of the connections observed in the present study.

Table 1
Neurochemical Phenotype and Projection Target of VTA Cells Synaptically Innervated by VACHT+ Terminals in the Rat

Tissue Labeled for TH	Dendrites labeled for:			
	Unlabeled	TH	FG	FG+TH
Retrograde Transport from NAc				
total VACHT+ terminals	269			
total dendritic contacts*	228 (85%)			
total synapses	123 (54%)	46 (37%)	52 (42%)	4 (3%) 21 (17%)
asymmetric synapses	96 (78%)	31 (32%)	42 (44%)	3 (3%) 20 (21%)
symmetric synapses	27 (22%)	15 (56%)	10 (37%)	1 (4%) 1 (4%)
Retrograde Transport from PFC				
total VACHT+ terminals	277			
total dendritic contacts	226 (82%)			
total synapses	80 (35%)	62 (78%)	12 (15%)	3 (4%) 3 (4%)
asymmetric synapses	51 (64%)	39 (76%)	10 (20%)	1 (2%) 1 (2%)
symmetric synapses	29 (36%)	23 (79%)	2 (7%)	2 (7%) 2 (7%)

Tissue Labeled for GABA	Dendrites labeled for:			
	Unlabeled	GABA	FG	FG+GABA
Retrograde Transport from NAc				
total VACHT+ terminals	214			
total dendritic contacts	147 (69%)			
total synapses	70 (48%)	46 (66%)	14 (20%)	3 (4%) 7 (10%)
asymmetric synapses	52 (74%)	37 (71%)	6 (12%)	3 (6%) 6 (12%)
symmetric synapses	18 (26%)	9 (50%)	8 (44%)	0 (0%) 1 (6%)
Retrograde Transport from PFC				
total VACHT+ terminals	261			
total dendritic contacts	169 (65%)			
total synapses	62 (37%)	35 (57%)	21 (34%)	4 (7%) 2 (3%)
asymmetric synapses	43 (69%)	29 (67%)	12 (28%)	2 (5%) 0 (0%)
symmetric synapses	19 (31%)	6 (32%)	9 (47%)	2 (11%) 2 (11%)

* dendritic contacts include synapses and appositions